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THE MECHANISM OF TRICHLOROETHYLENE OXIDATION BY TOLUENE DIOXYGENASE: IMPLICATIONS FOR BIOREMEDIATION

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ABSTRACT

The oxidation of trichloroethylene (TCE) by toluene dioxygenase *in vivo* and *in vitro* has been investigated. In a previous study, the initial rate of TCE oxidation by *Pseudomonas putida* F1 declined rapidly. This was shown *in vivo*, in the present study, to be due to toluene dioxygenase-dependent activation of TCE to produce reactive intermediates. Carbon-14 label from metabolism of radioactive TCE was incorporated into proteins, small molecules, DNA, RNA, and lipid. Alkylation of proteins, including toluene dioxygenase, caused metabolic poisoning and the loss of TCE-degrading ability. *P. putida* was able to recover from TCE-mediated cytotoxicity. Toluene dioxygenase enzyme components were purified from recombinant *E. coli* strains by the use of conventional chromatographic methods. The major products of TCE oxidation by purified toluene dioxygenase have been identified as formic acid and glyoxylic acid. An additional minor product was shown to result from the alkylation of reduced pyridine dinucleotide (NADPH) by a reactive TCE intermediate generated by the toluene dioxygenase catalyzed reaction. Experiments with ^{14}C-TCE showed that enzyme inactivation was due to non-specific alkylation of the proteins by diffusible reactive intermediates. A recombinant *E. coli* expressing cloned toluene dioxygenase genes oxidized TCE. The rate of TCE oxidation by the *E. coli* strain was slower than that of *P. putida* F1 but the rates were sustained for a longer time. The use of recombinant strains or gratuitously induced *P. putida* F1 could have important implications for bioremediation. Furthermore, a greater understanding of the mechanism of toluene dioxygenase inactivation will be crucial for developing optimum systems for TCE-biotreatment.

(a) Objectives of the research effort

It was the overall objective of this research to investigate the catalytic properties of toluene dioxygenase with trichloroethylene (TCE) as a substrate. Knowledge obtained about this oxidative degradative reaction impacts the designing of processes for bioremediation of TCE contaminated environments.

The specific objectives of the research project are listed below in the order in which they were investigated.

1. The products of TCE oxidation by toluene dioxygenase were determined.
2. Purified enzyme components were used for kinetic and radioisotope studies to determine if TCE is a suicide substrate for toluene dioxygenase.
3. The biochemical basis of the rapid fall off in *in vivo* TCE degrading activity in *P. putida* F1 was investigated.
4. The substrate and inducer specificity of toluene dioxygenase was explored.

(b) Report on the research effort

The oxidation of TCE by toluene dioxygenase *in vivo* and *in vitro* has been investigated. For clarity of presentation, *in vivo* studies will be discussed first, followed by the *in vitro* studies. In the course of these experiments, all of the objectives enumerated above have been addressed.

1. *In vivo* studies

Initial *in vivo* studies were conducted to better understand the observed rapid fall off in TCE degrading activity in *P. putida* F1. It was determined that TCE is metabolically activated by toluene dioxygenase to produce toxic effects in *P. putida* F1. Cytotoxicity was indicated by growth inhibition and by the covalent modification of cellular molecules in *P. putida* F1 exposed to [^{14}C] trichloroethylene. With a toluene dioxygenase mutant, neither growth inhibition nor alkylation of intracellular molecules was observed.

It was established that reactive intermediates generated during the oxidation of TCE by toluene dioxygenase reacted non-specifically within the cell. Carbon-14 label from radioactive TCE was incorporated into protein, small molecules, DNA, RNA, and

lipid following TCE oxidation for 10 min by *P. putida* F1. Most (68%) of the radiolabel was incorporated into the protein fraction. Fast protein liquid chromatography (FPLC) revealed that many proteins contained covalently bound radiolabel, including the toluene dioxygenase components. Treatment of the protein fraction with 6N HCl for 96 h released two radioactive materials that comigrated with authentic glyoxylic acid and formic acid by analysis using high-pressure liquid chromatography (HPLC). Glyoxylic acid and formic acid may be derived from glyoxyl chloride and formyl chloride, respectively. Both acyl chlorides are candidates for reaction with nucleophilic amino acid side chains in proteins as well as other reactive groups in DNA and RNA. This is addressed further below in the section on *in vitro* experiments.

Cellular toxicity caused by TCE metabolism led to a diminished growth rate of *P. putida* F1. This observation suggested that the alkylation of macromolecules might be partially counterbalanced by the turnover and resynthesis of critical biomolecules. Further experiments confirmed this. *P. putida* F1 could completely recover from the toxic effects of TCE within 3 h, or two doubling times, after removal of TCE. These data indicate that bioremediation systems using *P. putida* F1 or related organisms should be cycled with and without TCE to regenerate full metabolic capabilities during a biotreatment process.

The issue of enhanced TCE biodegradation was also addressed by cloning *P. putida* F1 toluene dioxygenase genes into *Escherichia coli*. These studies were conducted in a collaboration between my laboratory and that of Professor David Gibson who is supported by grant AFOSR-88-6225 from the U.S. Air Force Office of Scientific Research. In that joint study, recombinant *E. coli* strain JM109 (pDTG601) expressed toluene dioxygenase and degraded TCE. However, TCE oxidation by toluene dioxygenase proceeded differently in *E. coli* than in *P. putida*. Whereas *P. putida* F1 catalyzed a rapid initial rate of TCE degradation that declined rapidly, *E. coli* oxidized TCE at a slower but sustained rate of 6 h. The observation that TCE oxidation rates are more sustained in *E. coli* could reflect less damage to metabolism in this organism. Thus, the matching of a biodegradative enzyme activity with the proper host

strain might be an important theme in the use of recombinant DNA technology in bioremediation.

The substrate and inducer specificity of toluene dioxygenase has been investigated. Compounds that serve as both substrates and inducers include benzene, toluene, ethylbenzene, styrene, phenylacetylene, fluorobenzene, chlorobenzene, bromobenzene, anisole, phenol, *o*-cresol, benzthiophene, and benzonitrile. A few compounds investigated induced toluene dioxygenase but are likely not enzyme substrates. Two of these compounds, p-dioxane and tetrahydrofuran, are highly water soluble and relatively non-toxic. They might thus be eligible to be used as gratuitous inducers of toluene dioxygenase in bioremediation applications.

2. *In vitro* studies

A rigorous investigation of toluene dioxygenase reaction products and inactivation mechanisms necessitated the use of purified enzyme components. In the past, all three toluene dioxygenase components (Rd_{TOL}, Fd_{TOL}, ISP_{TOL}) have been purified from *P. putida* F1 by standard chromatographic techniques. These methods are feasible, but arduous, given the notorious instability of the purified protein components. In this context, novel approaches have been developed to obtain protein components for reconstitution of *in vitro* toluene dioxygenase activity. We have utilized *E. coli* JM109 (pDTG601a), kindly provided by Professor David Gibson of the University of Iowa, to obtain ISP_{TOL} in high yield. Strain *E. coli* JM109 (pDTG614) was used to obtain sufficient quantities of Fd_{TOL}. Lastly, we utilized spinach ferredoxin reductase in place of ferredoxin $reductase_{TOL}$.

In initial experiments, all three purified protein components and NADPH were required to oxidize TCE. This was expected based on analogous experiments with toluene and indicated that all three proteins were free of contamination.

Investigations into the products of TCE oxidation by toluene dioxygenase have been conducted. From the chemical modification studies described above, acyl chlorides were anticipated as TCE oxidation products. Methods to detect organic acids derived from hydrolysis of the acyl chlorides were described in the original research

proposal. These protocols proved to be cumbersome leading to the development of a superior procedures using high pressure liquid chromatograph (HPLC) fitted with a Bio-Rad Aminex HPX-87H column. The HPLC method cleanly separated trichloroacetic acid, dichloroacetic acid, glyoxylic acid, formate acid, and trichloroacetaldehyde. By starting with ^{14}C-TCE and chromatographing the oxidation products, we could identify the organic acids produced by toluene dioxygenase via co-chromatography with standard acids. We have observed the formation of formic acid, glyoxylic acid, and a compound that migrated in between these identified products. Further studies indicated that the intermediate peak corresponded to a modified reduced pyridine nucleotide (NADPH) which was present in the reaction mixture to provide reducing equivalents to toluene dioxygenase. It is likely to result from alkylation of NADPH by formyl chloride or glyoxyl chloride intermediates generated during the course of the reaction. Repeated efforts to detect phosgene, carbon monoxide, or trichloroacetaldehyde in the reaction mixtures were negative.

Next, experiments were conducted to determine the reaction stoichiometry, to insure a rigorous accounting of the reactants and products. The results of these studies are summarized in the table below.

A. Reactants consumed and non-volatile products formed

Reactants (nmol oxidized)		Non-Volatile Products	
Trichloroethylene	NADPH	nmol formed	% of TCE oxidized
3.46 ± 0.30(5)	27.05 ± 5.3(5)	2.42 ± 0.21(5)	70

B. Total products formed (% of TCE consumed)

Acidic Products			Volatile Products		Modified Protein
Formate	Glyoxylate	NADPH-Adduct	Carbon Monoxide	Carbon Dioxide	
47	17	6	<1	<5	1

In the experiments, 70% and 71% of the TCE oxidized was accounted for as acidic products and modified protein (ie. ^{14}C-label incorporated into the protein fraction). It is thought that the HPLC conditions, using an acidic eluant, might result in some losses of formate during chromatography.

The 1% of ^{14}C-label incorporated into protein was significant with regard to the mechanism of inactivation of toluene dioxygenase during TCE oxidation. Chromatography of the protein fraction through Sephadex G-25 indicated that the radio-label was covalently attached. Further chromatographic separation of the protein components and scintillation counting yielded data consistent with non-specific reaction of reactive intermediates with all of the proteins. These data are important as they suggest that the enzyme systems can be protected *in vivo* by intracellular nucleophiles that might intercept reactive intermediates. Furthermore, the results reported here suggest a possible explanation for the apparent greater stability of TCE oxidation activity in *E. coli* than in the native *Pseudomonas* expressing toluene dioxygenase. A possible role of recombinant DNA technology would be to provide the most suitable host organism for protecting against reactive intermediates generated during TCE oxidation.

(c) Collaborations with other laboratories

We have conducted formal collaborations with:

Dr. David T. Gibson
Department of Microbiology
The University of Iowa
Iowa City, IA 52242

Professor Gibson is studying biotechnological approaches on the biodegradation of chlorobenzenes and trichloroethylene under grant AFOSR-88-6225. We conducted joint studies on using a recombinant *E. coli* strain to degrade TCE as described in the section, "Report on the research effort."

(d) Publication of research results

1. In refereed journals

   a. Wackett, L.P. and S.R. Householder. (1989) Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. *Appl. Environ. Microbiol.* 55:2723-2725.

   b. Zylstra, G.J., L.P. Wackett and D.T.. Gibson. (1989) Trichloroethylene degradation by *Escherichia coli* containing cloned *Pseudomonas putida* F1 toluene dioxygenase genes. *Appl. Environ. Microbiol.* 55:3162-3166.

   c. Li, S. and L.P. Wackett. (1992) Oxidation of trichloroethylene by toluene dioxygenase from *Pseudomonas putida* F1. *Biochem. Biophys. Res. Comm.* (to be submitted).

2. Book chapter

   Wackett, L.P. (1992) Dehalogenation reactions catalyzed by bacteria. In (A.M. Martin, ed.) *Biological Degradation of Wastes*, Elsevier Press, Essex, England (in press).

3. Abstracts

   a. Wackett, L.P. (1990) Dehalogenation of priority pollutants by bacterial enzymes and cofactors. Intnl. Symp. Environ. Biotechnol., Braunschweig, Germany.

   b. Wackett, L.P., Li Shuying and S.R. Householder. (1990) Biodegradation of trichloroethylene by oxygenases. Abstr. Soc. Environ. Tox. Chem. Ann. Meet., P248, p. 177.

(e) Verbal presentation of research results

1. National and international meetings

   a. International Symposium of Environmental Biotechnology, Braunschweig, West Germany, April, 1990. "Dehalogenation of Priority Pollutants by Bacterial Enzymes and Cofactors"

   b. American Society for Microbiology Annual Meeting, Anaheim, CA, May, 1990. "Oxidation of Non-growth Compounds by Oxygenases"

   c. SETAC 11th Annual Meeting, session organized by AFOSR, to be held November 11-15, 1990. "Biodegradation of Trichloroethylene by Oxygenases"

2. University departmental seminars

   a. Texas College of Osteopathic Medicine, February, 1990. "Bioremediation of Chlorinated Compounds"

   b. Michigan State University, NSF Microbial Ecology Center, May, 1990. "Bacterial Cleavage of Carbon-Chlorine Bonds by Reductive, Hydrolytic, and Oxidative Mechanisms."

c. University of Colorado, Department of Chemistry and Biochemistry, November, 1990.
"Dehalogenation of Organohalide Pollutants by Bacterial Enzymes and Coenzymes."

d. Southern Illinois University, March, 1990
"Dehalogenation of Organohalide Pollutants by Bacterial Enzymes and Coenzymes."

e. University of Texas at Austin, September, 1991
"Metabolism of Halogenated Organic Compounds by Bacterial Enzymes and Coenzymes."

In all of the presentations indicated above, the generous support of the Air Force Office of Scientific Research was acknowledged on a slide at the end of the talk.

f. Inventions or patent disclosures

None